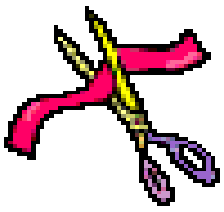
ENZYMES IN CLONING
PART I



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Cloning - a definition

- From the Greek - klon, a twig
- An aggregate of the asexually produced progeny of an individual;a group of replicas of all or part of a macromolecule (such as DNA or an antibody)
- An individual grown from a single somatic cell of its parent & genetically identical to it
- *Clone: a collection of molecules or cells, all identical to an original molecule or cell*

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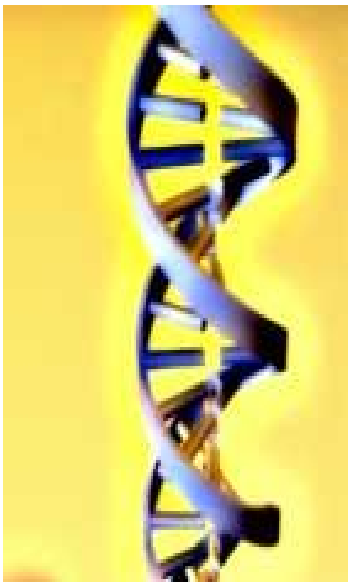
Different types of Cloning

1. Reproductive Cloning
2. Therapeutic Cloning
3. Recombinant DNA Technology or DNA Cloning



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DNA CLONING

A method for identifying and purifying a particular DNA fragment (clone) of interest from a complex mixture of DNA fragments, and then producing large numbers of the fragment (clone) of interest.

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What is genetic engineering

- Genetic engineering, also known as recombinant DNA technology, means altering the genes in a living organism to produce a Genetically Modified Organism (GMO) with a new genotype.
- Various kinds of genetic modification are possible: inserting a foreign gene from one species into another, forming a transgenic organism; altering an existing gene so that its product is changed; or changing gene expression so that it is translated more often or not at all.

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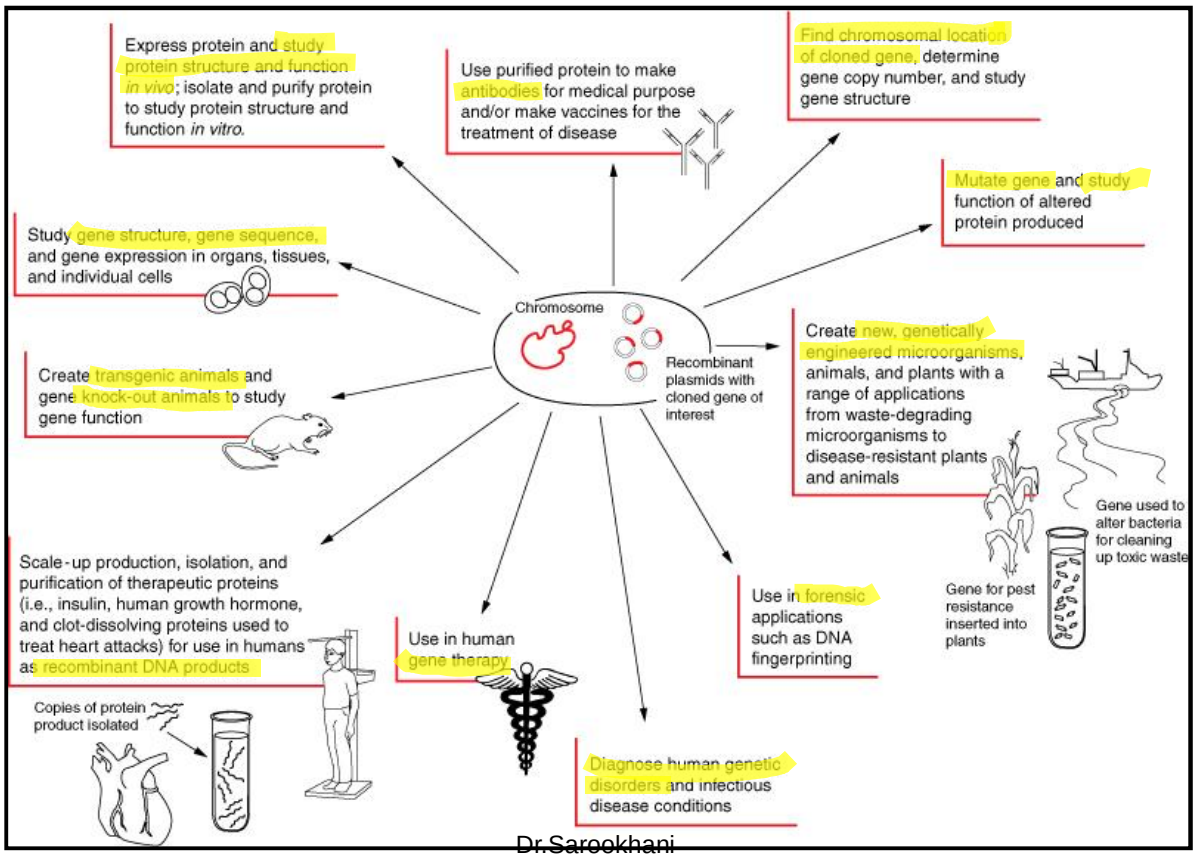

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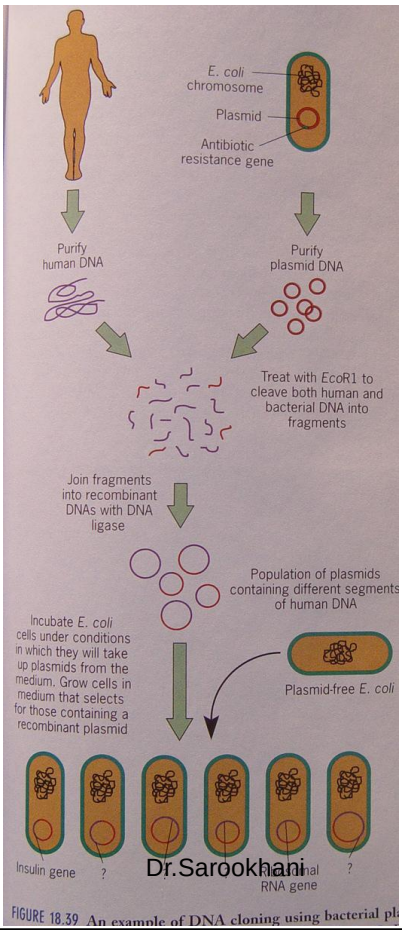
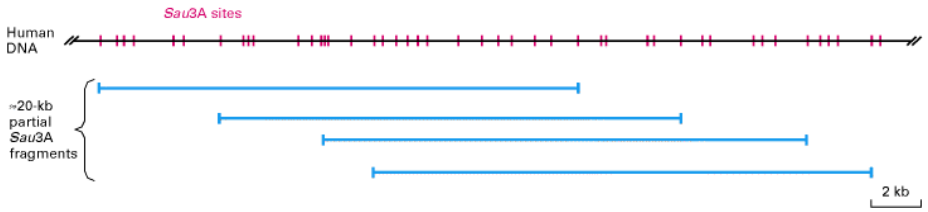


FIGURE 18.39 An example of DNA cloning using bacterial pl

Genomic Library



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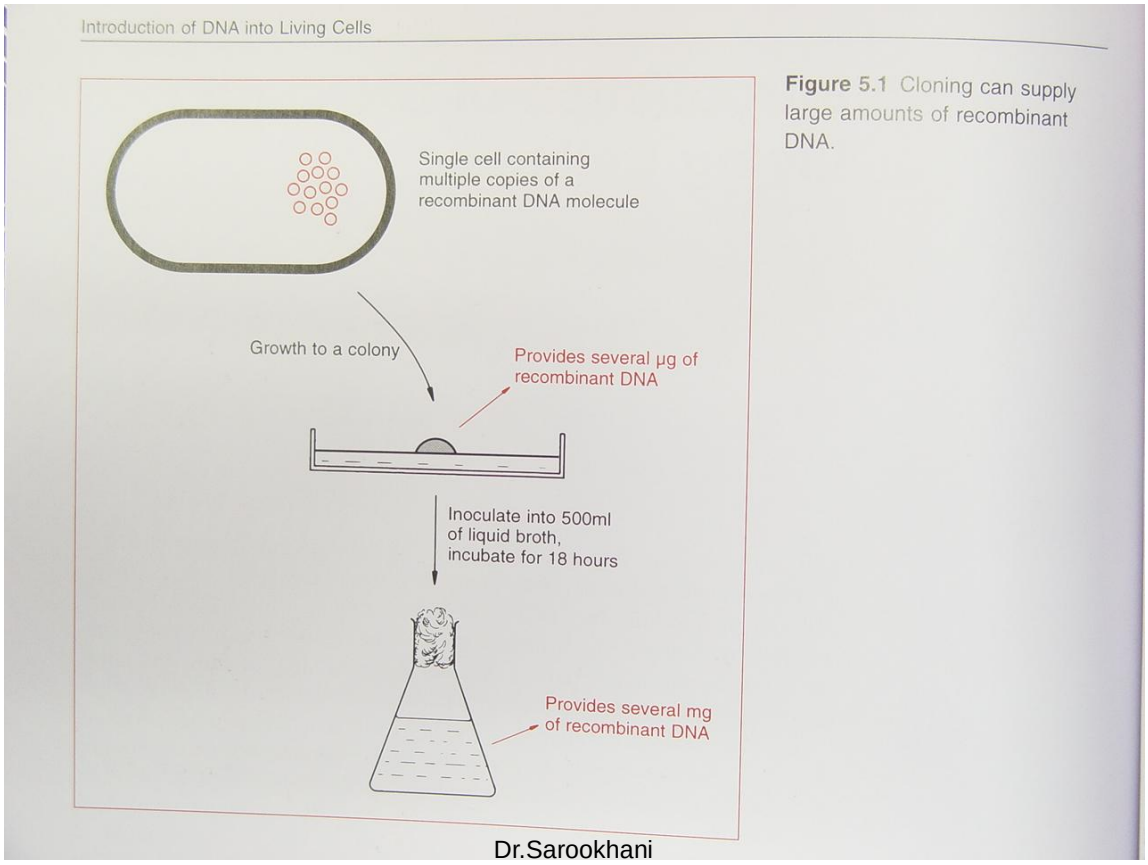

Figure 5.1 Cloning can supply large amounts of recombinant DNA.

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Basic steps in genetic engineering

1. Isolate the gene
2. Insert it in a host using a vector
3. Produce as many copies of the host as possible
4. Separate and purify the product of the gene

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Cloning Tools

- Restriction endonucleases
- Ligase
- Vectors
- Host
- Methods for introducing DNA into a host cell

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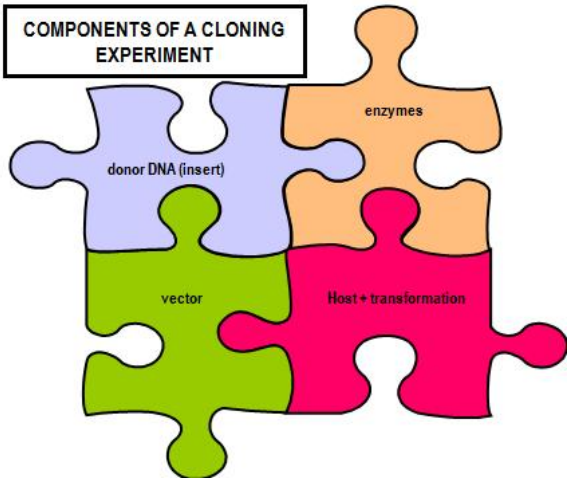

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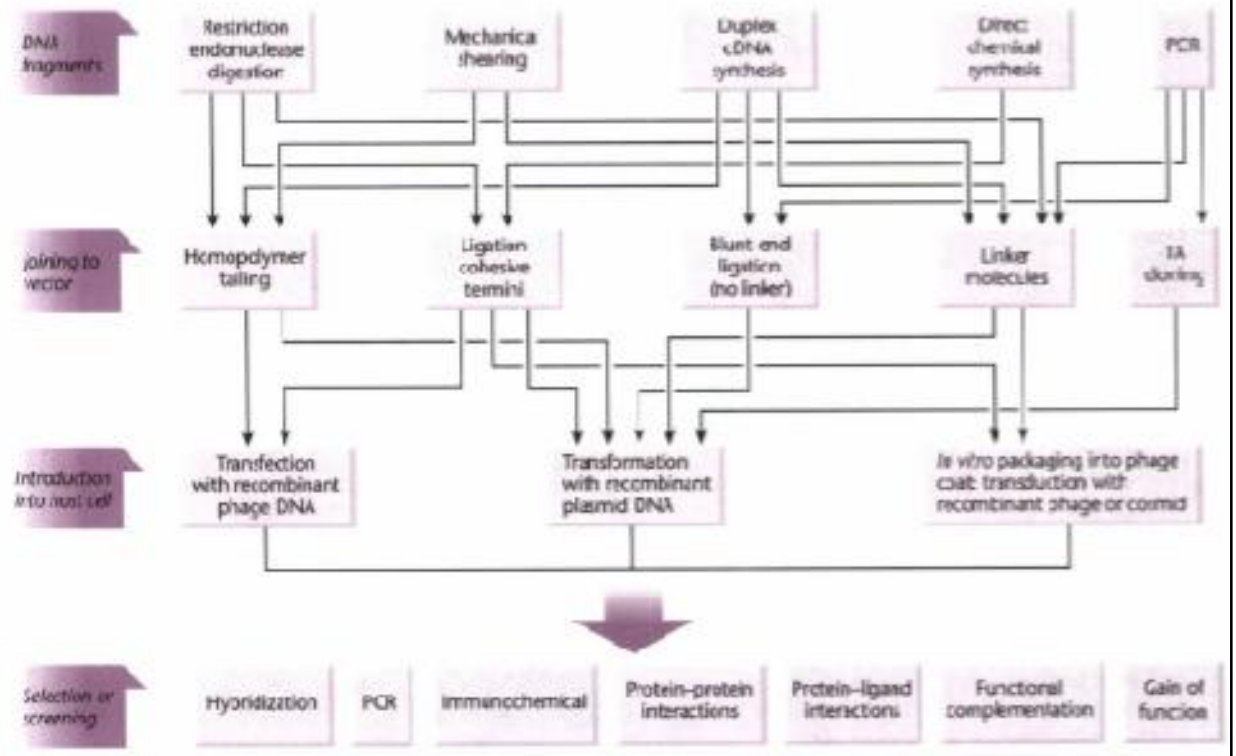

Fig. 6.1 Generalized overview of cloning strategies, with favored routes shown by arrows. Note that in cell-based cloning strategies DNA fragments are initially generated and cloned in a non-specific manner, so that screening for the desired clone is carried out at the end of the process. Conversely, when specific DNA fragments are obtained by PCR or direct chemical synthesis, there is no need for subsequent screening.

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WAYS OF GENERATING DNA FRAGMENTS

1. Non-specific generation of truly random fragments (by mechanical shearing or digestion with non-specific nucleases)
2. Through reverse transcription of mRNA into DNA
3. Highly specific amplification of a chosen piece of DNA by PCR
4. The use of synthetic DNA
5. *Restriction endonucleases digestion*

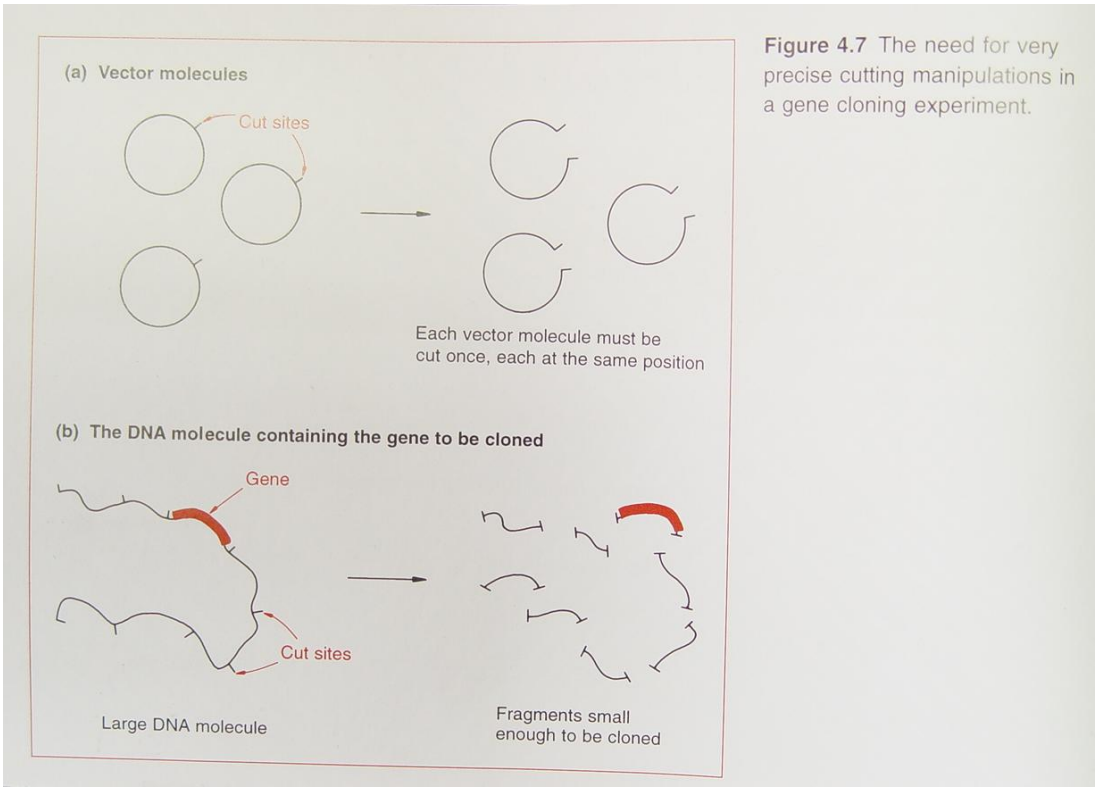

Figure 4.7 The need for very precise cutting manipulations in a gene cloning experiment.

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RESTRICTION ENDONUCLEASES

- Host-controlled restriction and modification phenomenon
  - Some strains of *E. coli* were immune to bacteriophage infection
  - In many bacterial species
  - Defense mechanism against foreign DNA

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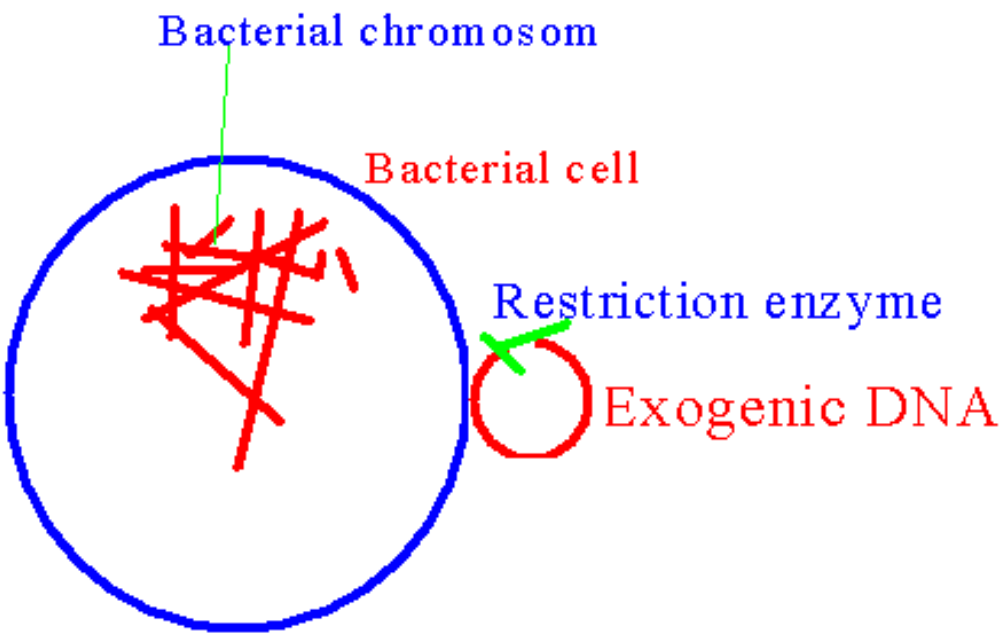

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HOST-CONTROLLED RESTRICTION AND MODIFICATION

2 components

1. Restriction endonuclease (Restriction Enzyme) R.ENase
2. Methylase (A or C) (Methylation Enzyme (M.ENase)

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RESTRICTION ENDONUCLEASES

- Enzymes
- Recognizes a short, symmetrical DNA sequence
- Hydrolyzes/cuts the DNA backbone in each strand
  - Specific site within that sequence
  - Foreign DNA is degraded into short fragments

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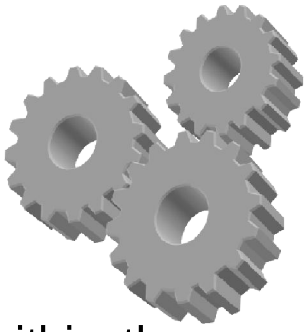
METHYLASE



- Modification enzyme
  - Adds a methyl group (C or A) within the same recognition sequences in the cellular DNA
    - Resistant to degradation by the endonuclease

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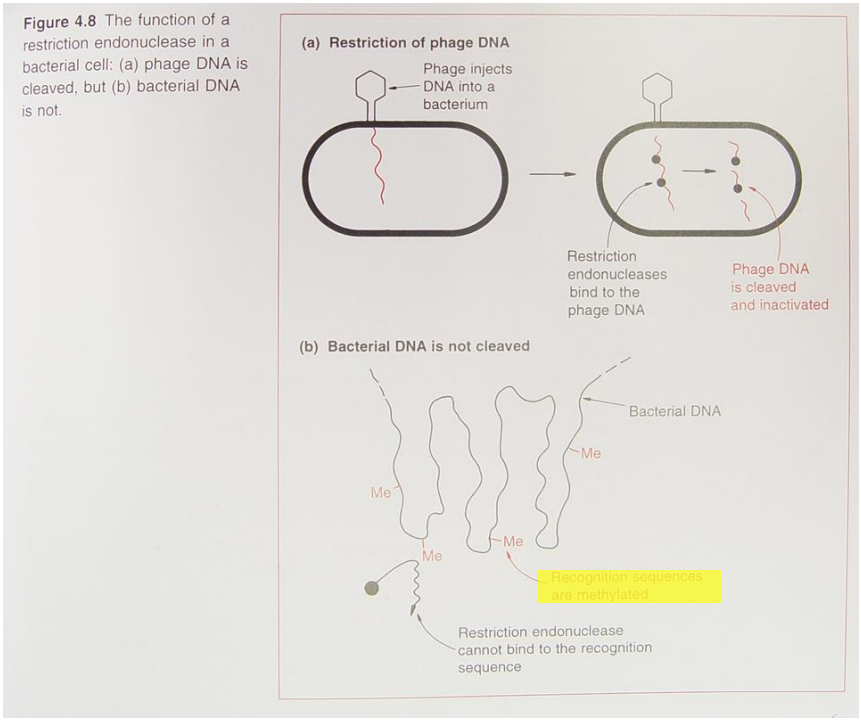

Figure 4.8 The function of a restriction endonuclease in a bacterial cell: (a) phage DNA is cleaved, but (b) bacterial DNA is not.

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Table 4.1 The recognition sequences for some of the most frequently used restriction endonucleases.

Enzyme	Organism	Recognition sequence[a]	Blunt or sticky end
*Eco*RI	*Escherichia coli*	GAATTC	Sticky
*Bam*HI	*Bacillus amyloliquefaciens*	GGATCC	Sticky
*Bgl*II	*Bacillus globigii*	AGATCT	Sticky
*Pvu*I	*Proteus vulgaris*	CGATCG	Sticky
*Pvu*II	*Proteus vulgaris*	CAGCTG	Blunt
*Hin*dIII	*Haemophilus influenzae* R_d	AAGCTT	Sticky
*Hin*fI	*Haemophilus influenzae* R_f	GANTC	Sticky
*Sau*3A	*Staphylococcus aureus*	GATC	Sticky
*Alu*I	*Arthrobacter luteus*	AGCT	Blunt
*Taq*I	*Thermus aquaticus*	TCGA	Sticky
*Hae*III	*Haemophilus aegyptius*	GGCC	Blunt
*Not*I	*Nocardia otitidis-caviarum*	GCGGCCGC	Sticky
*Sfi*I	*Streptomyces fimbriatus*	GGCCNNNNNGGCC	Sticky

[a] The sequence shown is that of one strand, given in the 5′ to 3′ direction. 'N' indicates any nucleotide. Note that almost all recognition sequences are palindromes: when both strands are considered they read the same in each direction, for example:

*Eco*RI
5′–GAATTC–3′
3′–CTTAAG–5′

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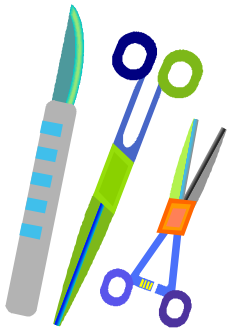
RESTRICTION ENDONUCLEASES

2. Part of the restriction-modification defense mechanism against foreign DNA
3. Basic tools of gene cloning



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RESTRICTION ENDONUCLEASES

3 types

Type I

Type II

Type III

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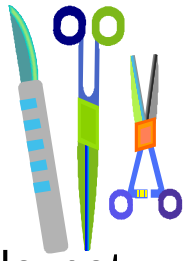
TYPE I REs



- recognize specific sequences in DNA but do not cut them
- it tracks along the DNA for a variable distance (1 kb⟶5 kb)
  - breaking the DNA strand
  - random
    - do not generate specific fragments
- Requirement: ATP, $Mg^{2+,}$ S-adenosyl methionine (S-AM)

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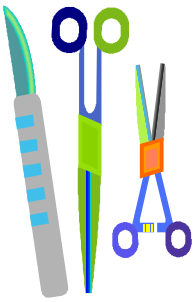
TYPE III REs



- cut at 24-25 bp on 3’ side of specificity site
- Requirement: ATP, Mg^{2+}, (S-adenosyl methionine)

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- آنزيم هاي R-M نوع III:
- . اين آنزيم هاتوالي هاي كوتاه غير پاليندرم را شناسايي كرده و DNA را از محلهاي معين (درصورتيكه درجايگاه شناسايي تغييري ايجادنشده باشد) برش ميدهند

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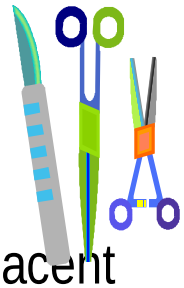
TYPE II REs



- commonly used in cloning
- recognize and cut within (or immediately adjacent to) specific target sequences
  - generate specific fragments
- a small number
  - cut the DNA at a defined distance (usually only a few bases) away from the recognition site
  - limited applications
- requirement: Mg^{2+}

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آنزيم هاي R-M نوع II :

- اين آنزيمها داراي فعاليت R.ENase و M.Mtase جداگانه ميباشند
- R.ENase يک توالي نوکلئوتيدي اختصاصي را روی رشته DNA شناسایی میکند و ان را هضم می کند
- M.Mtase همان توالی مشابه را شناسايي ميکنند و نوکلئو تید A يا C را متیله میکند درنتیجه انزیم R.ENase قادر به هضم DNA نمی باشد

- دو آنزيم R.ENase و M.Mtase اصطلاحا" Cognate همديگر ميباشند

- آنزيم هاي نوع IV:

- R.ENase و M.Mtase توالي هاي ناقرينه را شناسايي ميكنند . R.Enase در حضور Mg2+در فاصله معيني از جايگاه شناسايي, DNA هدف را برش ميدهد

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CHARACTERIZATION AND IDENTIFICATION

1. The name of the organism from which they are obtained
2. Write in *italics*
   - The first letter of the genus
   - The first two letters of the species name
3. A suffix indicating the specific enzyme from that species

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- عناصر خارج كروموزومي مانند ويروس يا پلاسميد به همين صورت متعاقب اسم مذكور آورده ميشود مانند: EcoR

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- بعد از اسامی فوق یک عدد (با حروف رومانی) نوشته می شود که نشان دهنده تاخر و تقدم کشف ان انزیم در باکتری می باشد

:

EcoRI

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CHARACTERIZATION AND IDENTIFICATION

- Example:
  - *Pst*I from *Providencia stuartii*
  - *Hae*I, *Hae*II and *Hae*III, three different enzymes, with different specificities from *Haemophilus aegyptius*

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THE PRODUCT OF REs DIGESTION

1. Products with protruding ends known as cohesive or ‘sticky’ ends
   - Fragments with unpaired single-stranded sequences either at the 5’ or 3’ ends

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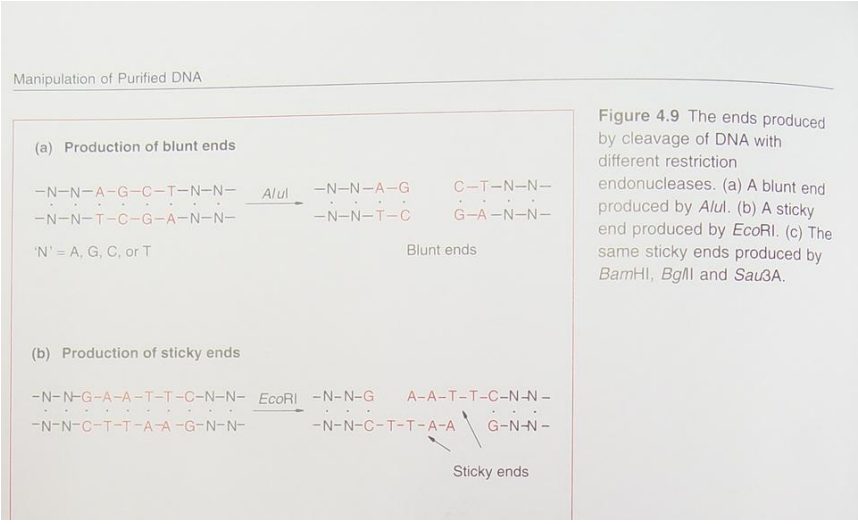

Figure 4.9 The ends produced by cleavage of DNA with different restriction endonucleases. (a) A blunt end produced by *Alu*I. (b) A sticky end produced by *Eco*RI. (c) The same sticky ends produced by *Bam*HI, *Bgl*II and *Sau*3A.

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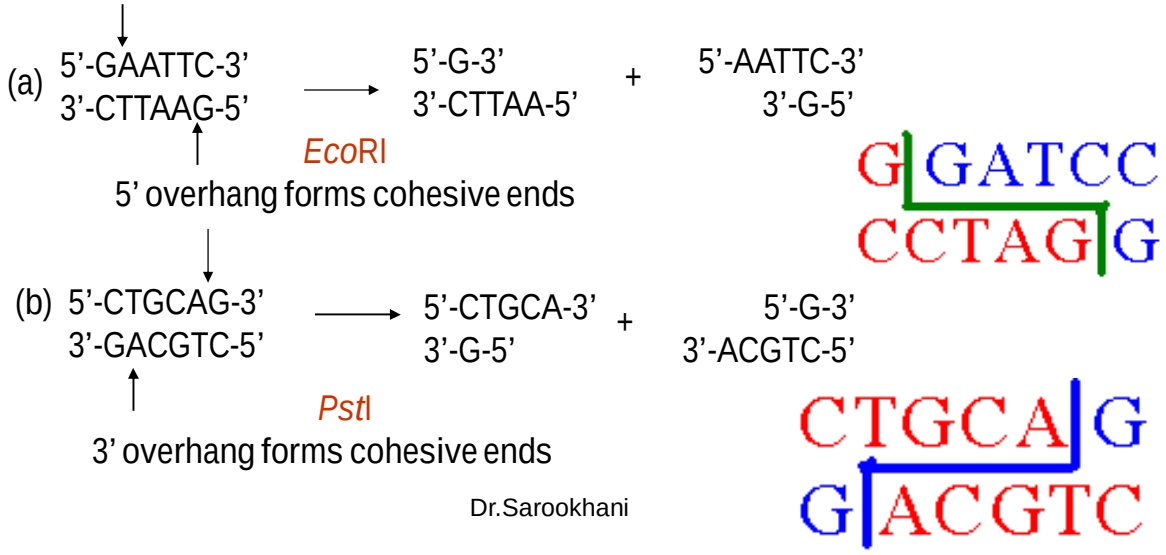
THE PRODUCT OF REs DIGESTION



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THE PRODUCT OF REs DIGESTION

2. Products with blunt ends

GAT|ATC
CTA|TAG

Eco321 (EcoRV)

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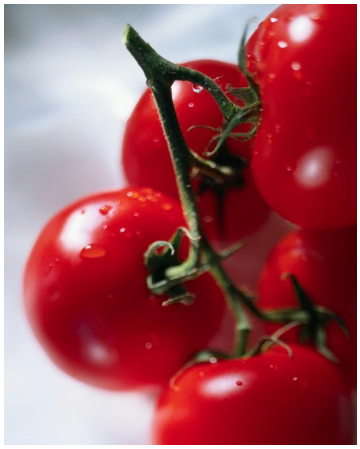
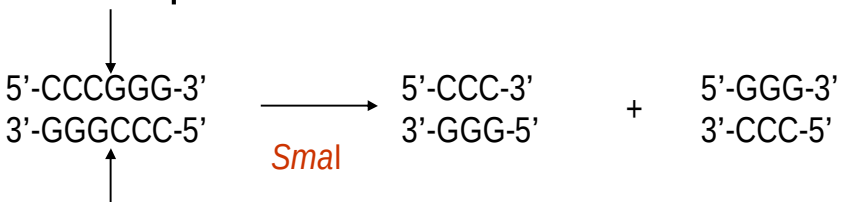


- Example



- Advantage: they can be joined to any other blunt-ended fragment
- Disadvantage: less efficiently ligated

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- جایگاه شناسایی انزیم های تیپ ۲ اکثرا" ۴ تایی یا شش تایی میباشد
- جایگاه شناسایی تعدادی از انها بصورت پالیندرم نمیباشد

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Enzymes	Recognition site	Number of bases	Ends generated	Source
*Eco*RI	G/AATTC	6	5' sticky	*Escherichia coli* RY13
*Bam*HI	G/GATCC	6	5' sticky	*Bacillus amyloliquefaciens* H
*Bgl*II	A/GATCT	6	5' sticky	*Bacillus globigii*
*Pst*I	CTGCA/G	6	3' sticky	*Providencia stuartii*
*Xma*I	C/CCGGG	6	5' sticky	*Xanthomonas malvacearum*
*Sma*I	CCC/GGG	6	blunt	*Serratia marcescens*
Sau3A	/GATC	4	5' sticky	*Staphylococcus aureus* 3A
*Alu*I	AG/CT	4	blunt	*Arthrobacter luteus*
*Not*I	GC/GGCCGC	8	5' sticky	*Nocardia otitidis-caviarum*
*Pac*I	TTAAT/TAA	8	3' sticky	*Pseudomonas alcaligenes*

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Nucleotide Specificity	Example	Frequency of Occurrence
Four	Alu I	256 (0.25 Kb)
Five	Nci I	1024 (1.0 Kb)
Six	EcoR I	4096 (4.1 Kb)
Seven	EcoO109I	16384 (16.4 Kb)
Eight	Not I	65536 (65.5 Kb)

Table 3.3 Some restriction endonucleases and their recognition sites.

Enzyme	Recognition sequence
4-base cutters	
*Mbo*I, *Dpn*I, *Sau*3AI	/GATC
*Msp*I, *Hpa*II	C/CGG
*Alu*I	AG/CT
*Hae*III	GG/CC
*Taq*I	ACGT/
6-base cutters	
*Bgl*II	A/GATCT
*Cla*I	AT/CGAT
*Pvu*II	CAG/CTG
*Pvu*I	CGAT/CG
*Kpn*I	GGTAC/C
8-base cutters	
*Not*I	GC/GGCCGC
*Sbf*I	CCTGCA/GG

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Many REs have the same recognition site and cut in the same place within that recognition sequence

BUT

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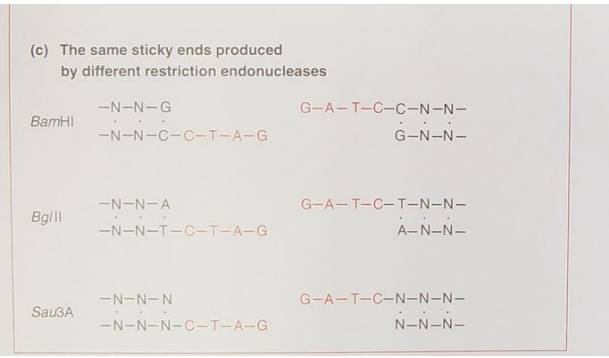
An enzyme that recognizes slightly different sequence, but produces the same ends is a isocaudomer.



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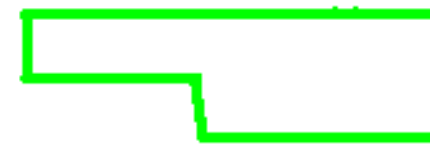
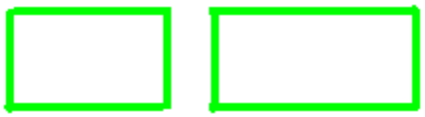
- ممکن است یک ترادف توسط چند انزیم شناسایی شود اما مدل برش انها باهم متفاوت باشد



SacI

GAGCTC
CTGGAG



Ecl136II

GAG CTC
CTG GAG

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Some REs recognize the same sequence but cut in a different position within that sequence

ISOSCHIZOMERS

The first enzyme to recognize and cut a given sequence is known as the prototype, all subsequent enzymes that recognize and cut that sequence are isoschizomers. Isoschizomers are isolated from different strains of bacteria and therefore may require different reaction conditions.

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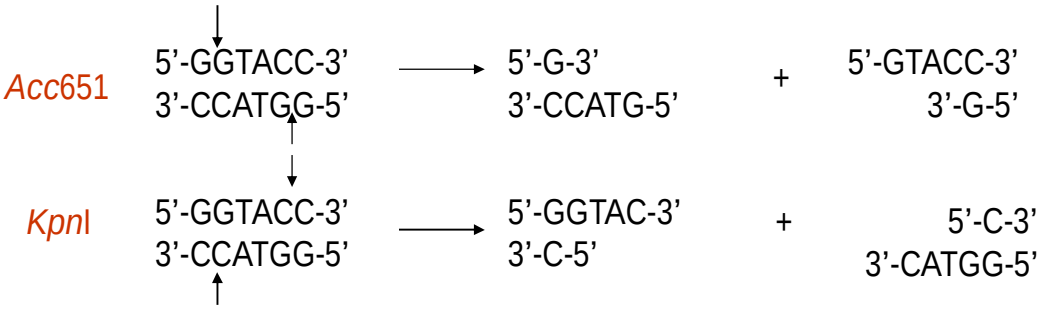
EXAMPLES



- *Acc*651 and *Kpn*I recognize the sequence GGTACC, but cut it at a different place
- Generate different sticky ends
- Option of obtaining virtually the same fragment of DNA but with different sticky ends that can be ligated to other fragments

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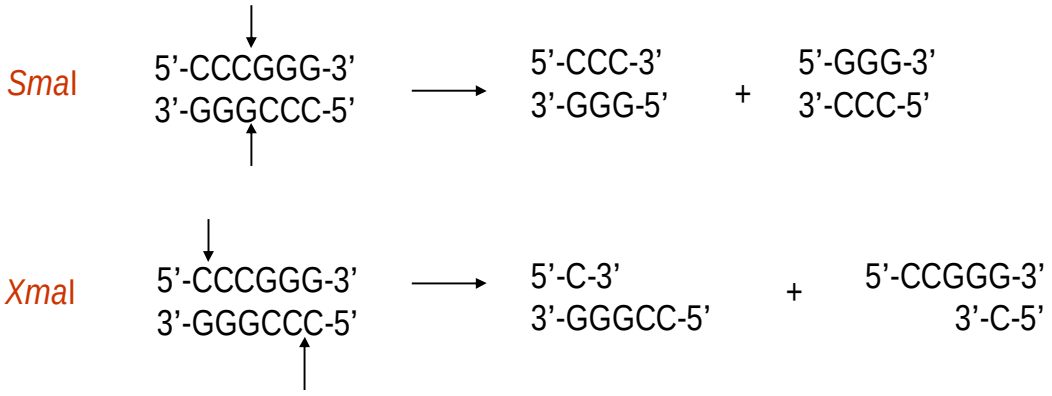
EXAMPLES



•*Xma*I cuts asymmetrically and produces sticky ends that can be ligated to other *Xma*I fragments, while *Sma*I will generate blunt-ended DNA fragments at the same site, allowing ligation to other blunt-ended DNA sequences

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An enzyme that recognizes the same sequence but cuts it differently is a neoschizomer. Neoschizomers are a specific type (subset) of Isoschizomers. For example, Sma I (CCC/GGG) and Xma I (C/CCGGG) are neoschizomers of each other.

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Star activity

بعضی از انزیمهای محدودگر در شرایطی (مانند زیاد بودن انزیم , طولانی بودن زمان انکوباسیون و.....) بجای اینکه شش نوکلئوتید را شناسایی کنند(eg.EcoR1) چهار نوکلئو تید وسط را شناسایی می کنند(eg.HindIII) و تعداد جایگاههای بیشتری را روی رشته شناسایی کرده و انرا هضم می کنند(مثل اینکه چندین آنزیم عمل کرده و چندین جایگاه ایجاد شده است)

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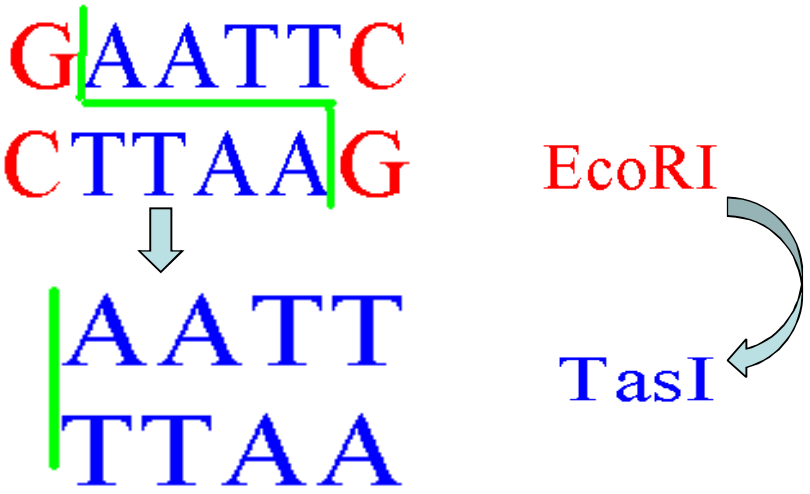
Star activity



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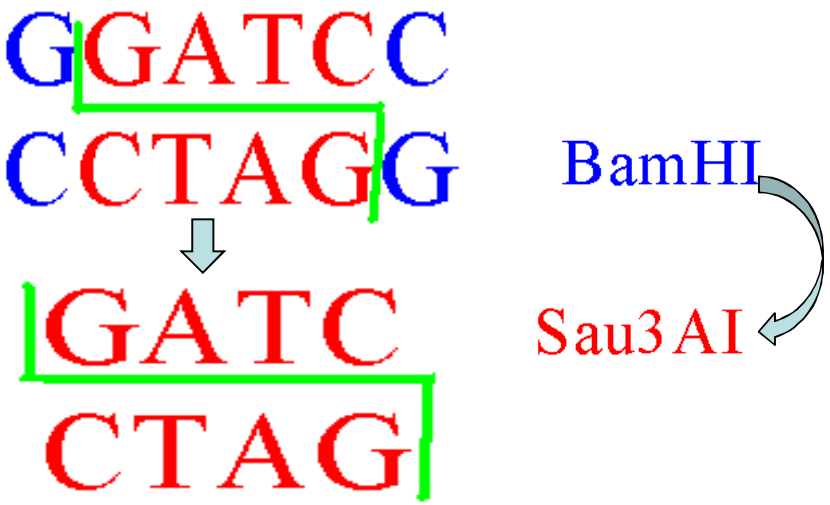
Star activity



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Star activity

- this phenomenon is the result of:
- prolonged incubation (over digestion),
- high enzyme concentration in the reaction mixture (over digestion),
- high glycerol concentration in the reaction mixture,
- presence of organic solvents, such as ethanol, dimethyl sulfoxide (DMSO) or dimethyl formamide (DMFA), in the reaction mixture,
- low ionic strength of the reaction buffer,
- suboptimal pH values of the reaction buffer,
- substitution of Mg^{2+} for other divalent cations, such as Mn^{2+} or Co^{2}

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How to distinguish between star activity and incomplete digestion?

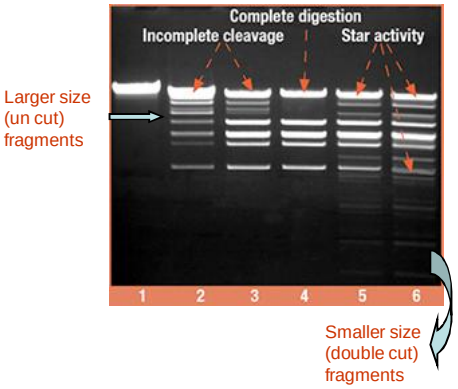

Figure 1. Enzyme star activity.
1 – Lambda DNA
2 – Lambda DNA incubated 1 hour with 0.15 u of EcoRI (incomplete cleavage)
3 – Lambda DNA incubated 1 hour with 0.4 u of EcoRI (incomplete cleavage)
4 – Lambda DNA incubated 1 hour with 1 u of EcoRI (complete digestion)
5 – Lambda DNA incubated 16 hours with 40 u of EcoRI (star activity)
6 – Lambda DNA incubated 16 hours with 70 u of EcoRI (star activity)

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تعریف واحد انزیم

- یک واحد انزیم مقدار انزیمی است که در مدت یک ساعت و در حرارت اپتیموم یک میکرو گرم DNA فاژ لامبدا را هضم کند

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- 2 µL of 10X buffer for
- 20 µL final volume

2 u for 2 µg DNA

2 U /4 u µL =0.5 µL

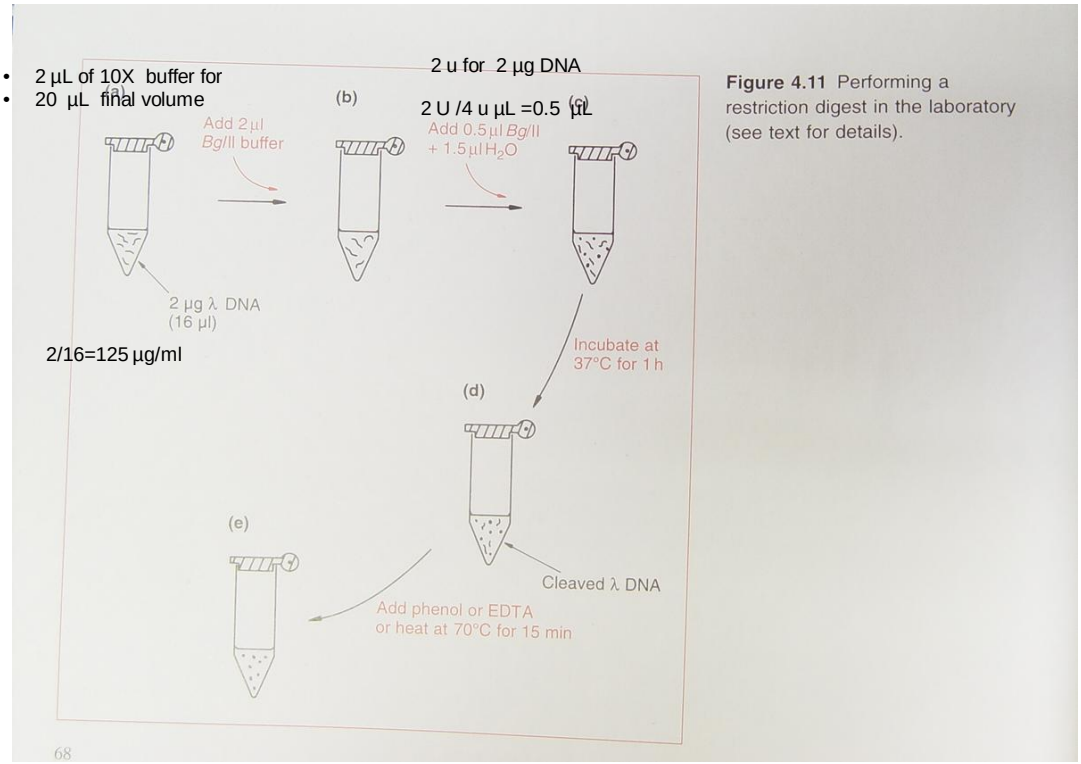

Figure 4.11 Performing a restriction digest in the laboratory (see text for details).

2/16=125 µg/ml

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- هضمDNA باآنزيم هاي محدودكننده (restriction digestion)

(كارها روي يخ انجام گيرد):

- ١-. مقدار پلاسميد را تخمين بزنيد (يك واحد آنزيم مقدار يك ميكرو گرم DNA را در حرارت مناسب در مدت يك ساعت هضم ميكندويك ميكروگرم DNA را در يك واكنشي به حجم ٢٠-٣٠ ميكروليتر با آنزيم هضم كنيد)
- ٢- باآب مقطر حجم آن رابه ١٨ ميكرليتر (يا هرحجم مورد نظر) برسانيد
- ٣- لوله واكنش را spin كنيد
- ٤- مقدار ٢ميكروليتر از 10x Reaction buffer به واكنش اضافه كنيد(غلظت نهايي بافر 1x باشد)
- ٥- با استفاده از سرسمپلر استريل و سرد مقدار آنزيم مورد نظر را به واكنش اضافه نماييد (اين مقدار آنزيم بايد در حجم نهايي در نظر گرفته شده باشد).
- ٦- با ضربه نوك انگشت واكنش را مخلوط كرده و سپس spin نماييد.
- ٧- مدت يك تا دوساعت در حرارت مناسب انكوبه كنيد
- مقدار ١٥ ميكروليتر از واكنش را با ٣ ميكرو ليتر loading buffer مخلوط كرده و روي ژل آگارز با درصد مناسب الكتروفورزنماييد (از Intact plasmid DNA بعنوان كنترل واكنش روي ژل دركنارنمونه الكتروفورز شود توجه : اگر DNA هضم نشده است آن را P.C.I extraction نموده و با الكل رسوب داده وواكنش را تكرار كنيد).

Table 4.2 A 10× buffer suitable for restriction of DNA with *Bgl*II.

Component	Concentration (mM)
Tris-HCl, pH 7.4	500
$MgCl_2$	100
NaCl	500
Dithiothreitol	10

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Restriction Enzyme Exercise

- You have the following enzymes in your freezer (DraII, AleI, BamHI, Hsp921, KpnI, SbfI, VspI) and you want to know if any of them cut your pcr product so you can save your lab money!
- This time paste your sequence into the window and select the following REN form the “Include” window (highlight the following enzymes, you will need the hold the shift button down to include more then one enzyme: DraII, AleI, BamHI, Hsp921, KpnI, SbfI, VspI
- Do any of the enzymes cut your sequence? If yes, which one? How many times does it cut? Where?

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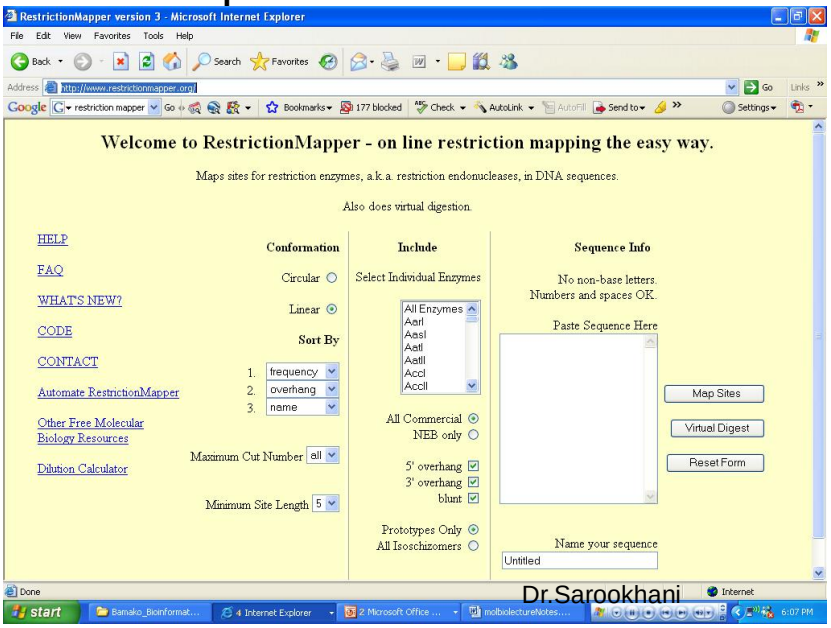
Restriction Enzyme Exercise

- Go to: http://www.restrictionmapper.org/
- Paste (cntrl v) your sequence into the window and click Map Sites



How many REN sites are found?

Will EcoRI cut your sequence?

How many times does ApoI cut?

I have amplified this gene and the PCR product is ~500 nt. I want to digest it into 2 aproxiamtely equal fragments, which enzyme would I use?

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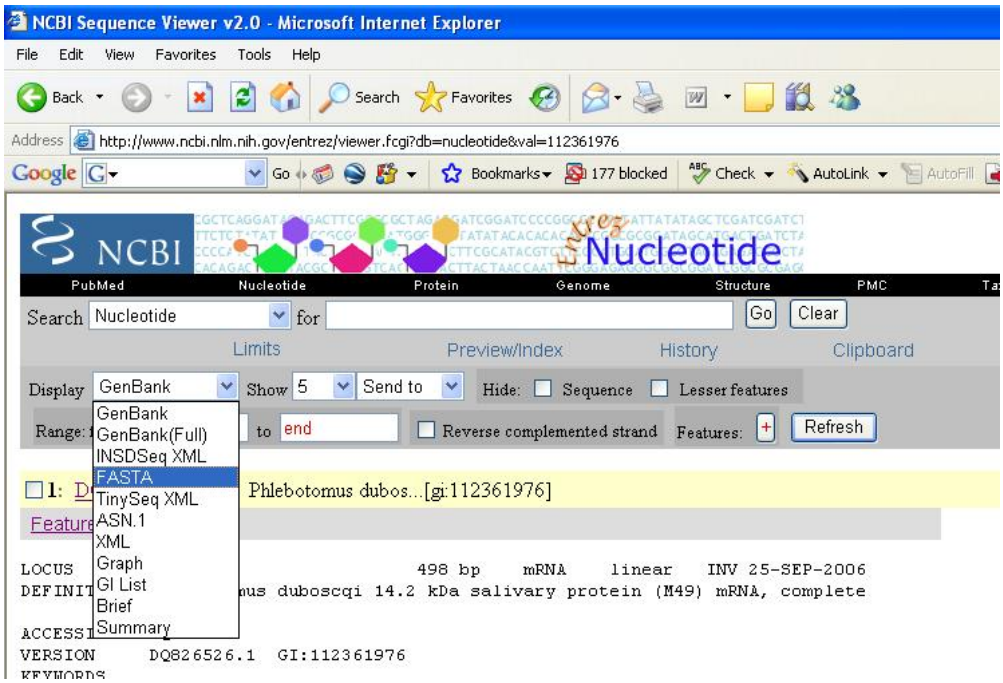

- Change the display to FASTA and then copy the sequence (ctrl c)

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